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# RESPIRATORY STUDIES ON ALLOXAN DIABETIC TISSUES

and

# OXYGEN CONSUMPTION OF MAMMARY TISSUE IN CANCER SUSCEPTIBLE MICE

by

LORNA GLEN SHAW
B.Sc. (Alberta)

Thesis
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The undersigned hereby certify that they have read and recommend to the School of Graduate Studies for acceptance, a thesis entitled "Respiratory Studies on Alloxan Diabetic Tissues" and "Oxygen Consumption of Mammary Tissue in Cancer Susceptible Mice" submitted by Lorna Glen Shaw, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

Professor

Professor

Date April 20, 1950. Professor

THE UNIVERSITY OF ALBERTA

# RESPIRATORY STUDIES ON ALLOXAN DIABETIC TISSUES
# and
# OXYGEN CONSUMPTION OF MAMMARY TISSUE IN CANCER SUSCEPTIBLE MICE

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE.

FACULTY OF ARTS AND SCIENCE

by
Lorna Glen Shaw.

EDMONTON, ALBERTA
1950

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# ACKNOWLEDGEMENT.

I wish to express my thanks to Dr. M.M. Cantor for his guidance of the work presented in Section I.

I wish to thank Dr. H.E. Rawlinson and Dr. J. Tuba for their guidance and assistance in the work presented in Sections II and III. I wish to thank Dr. H.E. Rawlinson also for his help with the staining, for translations of German articles and for the photographs of the slides. I would also like to thank Dr. H.B. Collier for his helpful criticism of the thesis.

I am indebted to Mr. R. Clelland for his willing assistance in the care of the rats. Also I wish to thank Mr. R. Hoare for the use of some of his animals and Mr. P. Beamont and Mr. A.G. Fairall for their help in a number of ways.

The work in Section I was conducted with the assistance of a grant to Dr. M.M. Cantor from the Division of Medical Research, National Research Council, Ottawa, (Project MP 178). The work in Sections II and III was conducted with the assistance of a grant to Dr. J. Tuba from the National Cancer Institute (Project 75).

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# SECTION I.

# RESPIRATORY STUDIES ON ALLOXAN DIABETIC TISSUES

# INTRODUCTION.

Changes in the metabolism of phosphorus containing compounds and in an enzyme which acts on phosphorus compounds, phosphatase, have been reported in alloxan diabetic rats. Elevation of serum alkaline phosphatase was reported by Cantor, Tuba and Capsey (4) and of liver phosphatase by Marsh and Drabkin (14).

The high serum alkaline phosphatase activity was thought to be due to increased fat metabolism since animals on a high-fat diet also exhibit high serum phosphatase levels and it is known that diabetic animals must use fat rather than carbohydrate as a source of energy. Marsh and Drabkin (13) have reported an inhibitory effect upon phosphorylation and oxygen consumption in kidney due to increased dephosphorylation. This was demonstrated by experiments in which a phosphatase preparation was added to kidney homogenates. Because oxidation and phosphorylation are so intimately associated it was thought that a study of the oxygen consumption and respiratory quotient of the various organs would give additional information and further elucidate the metabolic pattern in diabetes.

## Review of Literature.

Before discussing the work it is necessary to summarize what is known about diabetic metabolism.

The original work on experimental diabetes was done on depancreatized animals, although later phlorizin and more recently alloxan have been used. Results obtained on depancreatized and phloridzinized animals are complicated by other than diabetic symptoms. When the whole pancreas is removed a number of other factors besides insulin are being removed notably digestive enzymes including one (lipase) which helps digest fat. A lipotropic factor "lipocaic" is also thought to be secreted by the pancreas. Depancreatized animals only live a few months even when insulin is supplied. They die with extremely fatty livers (17).

Phloridzin, on the other hand, produces a renal diabetes which is accompanied by hypoglycemia. The action of phloridzin is apparently on the renal tubules where, according to the Lundsgaard Hypotheses (12), it prevents glucose reabsorption by inhibiting glucose phosphorylation within the kidney cells.

Alloxan, which causes selective necrosis of the Islets of Langerhans, produces the typical diabetic symptoms of hyperglycemia, glucosuria and polyuria.

The respiratory quotient (RQ) of the whole animal was studied as early as 1922 by Banting et al (2). They found that the RQ after 22 hours starvation was 0.85. If the animal was fed sucrose the RQ rose to 1.0 in 35 minutes and remained at this level for two hours. The RQ of the animal 48 hours after depancreatization was 0.63 and rose only to 0.78 in 1½ hours after feeding sucrose. If insulin was given with the sucrose the RQ in 30 minutes was 0.86 and 0.90 in 1½ hours. The RQ could sometimes be raised to 1.0 by this means.

Canzanelli et al (5) found no difference between the ability of normal and alloxan diabetic rat tissues (liver, kidney and brain) to oxidize glucose, pyruvate, lactate, succinate and glutamine.

The reaction known to be affected by insulin is:

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-phosphate} + \text{ADP} \ (6,16)$$

The reaction; Glycogen $\longrightarrow$ lactic acid, is not affected by insulin (16).

Since glucose cannot be used as a source of energy,fat and protein are used and this causes;

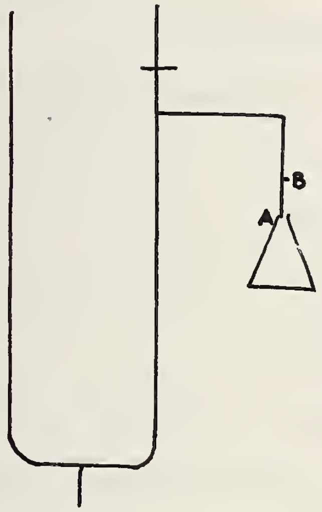
(1) Increased ketone formation by the liver and

(2) Increased protein deamination.

## METHODS.

The Test Animal: Adult, male, albino rats (Wistar Strain) were used. They were housed in all-metal cages and were given Purina Fox Checkers with tap water ad lib.

Calibration of Manometers: The oxygen consumption was measured by the Warburg Technique which involves the use of reaction vessels and manometers. The manometers and flasks are cleaned with potassium di-chromate and sulfuric acid, rinsed with water, alcohol and ether and dried at 37°C. The manometers are turned upside down and mercury is poured in at A (mercury is previously cleaned with dilute $HNO_3$). The amount of mercury is adjusted so that it rises in the right-hand column to 15 mm and in the side-arm to within approxiamately one inch of A. This spot is marked with a wax pencil (B). When the plugs are solid this can be made easier by putting a small piece of rubber tubing over the end A to hold a resevoir of mercury. The mercury is poured out into a weighed vessel.



The reaction vessel is filled with mercury until it contains enough to rise to B when it is fitted onto the manometer. This mercury is poured in with the rest and weighed to 0.05 g.. The temperature of the mercury is taken. This is repeated until two weighings agree to within 0.1 g. of each other.

Calculations:

$$\text{Volume in cmm.} = \frac{\text{weight in mg.}}{\text{density of Hg}}$$

Reagents.

(1) Manometer fluid; Brodie's solution is used which consists of;

500 ml. water
23 g. sodium chloride
5 g. sodium taurocholate
A few drops of alcoholic thymol.

A dye is added to this. Malachite green was tried but was not satisfactory since it faded within a week. Methyl violet was then used and is very satisfactory. To insert fluid into manometer the rubber tubing is filled with Brodie's solution and tightened onto the manometer.

10,000 mm. Brodie's solution = 760 mm. Hg

Density of fluid = 1.034

(2) Stopcock grease: Anhydrous lanolin was used but was not satisfactory since it tended to melt at 37°C. Fisher's 'Cello-seal' gives better results.

(3) Ringer's solution:

| | |
|---|---|
| 0.9 % NaCl (0.154 M) | 96 parts. |
| 1.15% KCl (0.154 M) | 2 parts. |
| 1.22% CaCl (0.11 M) | 2 parts. |

This is prepared once a week from stock solutions which are five times the above strength. Stock solutions keep from one to two months.

(4) 0.1 M Phosphate buffer:

35.8 g. $Na_2HPO_4 \cdot 12\ H_2O$

The pH is adjusted to 7.4 with normal HCl and then the solution is diluted to one liter. This buffer keeps indefinitely.

(5) Glucose: 10% solution. A drop of chloroform is added to prevent fermentation.

(6) Ringer-phosphate:

| | |
|---|---|
| Phosphate buffer | 1 part. |
| Ringer solution | 9 parts. |

(7) Ringer-phosphate-glucose:

| | |
|---|---|
| Ringer-phosphate | 100 ml. |
| Glucose solution | 2 ml. |

(8) Ringer-bicarbonate:

| | |
|---|---|
| 1.3% $NaHCO_3$ (0.154 M) | 16 ml. |
| Ringer solution | 84 ml. |

The sodium bicarbonate is gassed for one hour with carbon dioxide before the Ringer-solution is added. The Ringer-bicarbonate solution is gassed with 95% $N_2$:5% $CO_2$ for ten minutes before using.

## Measurement of Endogenous Respiration.

The animal is killed by decapitation and as quickly as possible the organ is removed and placed in ice-cold Ringer-solution. The organ is sliced (with a razor blade) as thinly as possible and the slices are placed in Warburg flasks in which 2 ml. of Ringer-phosphate or Ringer-phosphate-glucose had previously been placed. A fluted piece of #40 Whatman filter paper (2.0 x 2.2 cm.) is placed in the center well and 0.2 ml. of 20% KOH is added to it. The flasks are attached to the manometers and gassed with 100% oxygen for ten minutes. The manometers are then placed on a constant temperature bath (37°C) and shaken for 15 minutes to bring the flasks to the temperature of the bath and then read every ten minutes for an hour. A control vessel is run in which there is the same amount of fluid but no tissue. Its readings are subtracted.

Calculation:

$$Q_{O_2} = h\left(\frac{V_G \times \frac{273}{T} + V_F \times \alpha}{P_o}\right)$$

Where, K = part in brackets.
$V_G$ = volume of gas in manometer and flask in μl.
T = the absolute temperature of the water bath.
$V_F$ = the volume of the fluid.
$\alpha$ = solubility of gas in fluid (μl gas/ml. fluid, when gas is at pressure of one atmosphere at temperature T.)
Po = standard pressure in terms of manometer fluid.
h = the observed change in manometer reading in mm.

For any experiment everything but h is constant. The easiest way to calculate the rest of the equation is from a graph in which the volume of fluid in ml. is plotted against K. There is a straight line relationship.

The calculation of the endogenous respiration ($Q_{O_2}$) therefore involves multiplying K times the change in the manometer reading over a period of time and calculating it on an hourly basis. The $Q_{O_2}$ is expressed as μl. of oxygen/hour/mg. dry weight of tissue.

To obtain the dry weight, the slices are removed from the flasks after the experiment, placed on previously weighed watch glasses and dried at 110°C overnight.

The thickness of the slices is measured by dividing the square area of the slices in mm. into the dry weight in mg. times five. This was always less than the limiting thickness as worked out by Warburg (21) for liver slices.

## Measurement of Respiratory Quotient.

Four manometers and flasks are required; one as a control (#1), one for endogenous respiration (#2) and two more (#3)(#4) which have no KOH or filter paper in the inset but have 0.2 ml. of 3 M HCl in the sidearm. Into one of these (#3) the acid is tipped in at the start of the experiment and into the other (#4) the acid is tipped in at the end of the experiment. The one into which the acid is tipped at first gives the initial $CO_2$ in the medium. The changes in the manometer fluid of the last vessel are due to;

1. Oxygen consumption.
2. Carbon dioxide of respiration.
3. Initial carbon dioxide in the medium.

Calculation of the amount of carbon dioxide evolved ($X_{CO_2}$).

$$X_{CO_2} = h_4 - h_3 \left( \frac{K_{3CO_2}}{K_{4CO_2}} - \frac{X_{O_2}}{K_{4O_2}} \right) K_{4CO_2}$$

Where; $h_3$ = the observed change in the reading of manometer #3
$h_4$ = the observed change in the reading of manometer #4
$K_{3CO_2}$ = $CO_2$ constant for flask #3
$K_{4CO_2}$ = $CO_2$ constant for flask #4
$X_{O_2}$ = $Q_{O_2}$ calculated from flask #2

All manometer readings are divided by the dry weight before being used in the above calculations. The respiratory quotient is then the $X_{CO_2}$ divided by $X_{O_2}$.

## Measurement of Glycolysis.

Ringer-bicarbonate-glucose is used as the medium. Slices are placed in 2 ml. of medium in the flasks and gassed on the water bath with 5% $CO_2$:95% $N_2$ for twenty to thirty minutes. Manometers are read for thirty minutes. $Q_G^{N_2}$ referes to anaerobic glycolysis and is the µl. of carbon dioxide/ hour/ mg. dry weight of tissue.

For any further details see Manometric Methods (20).

## EXPERIMENTAL.

### 1. Normal and Diabetic Animals.

At first only the oxygen consumption of tissues of normal animals was measured. Later the RQ was also measured on a number of diabetic animals before the method was completely established. In table II an asterisk indicates the point at which change was made to the satisfactory method indicated above. RQ studies on normal tissue were all done by this method.

Diabetes was produced by a single injection of alloxan (160 mg./ Kg. body weight) to 24 hour starved rats. The animals were judged diabetic if their blood sugar exceeded 200 mg.%.

TABLE I.

Oxygen consumption ($Q=Q_{O_2}$) and RQ of normal, male rat liver and kidney.

| No. of rats. | Liver Q | Kidney Q | Liver RQ | Kidney RQ |
|---|---|---|---|---|
| 12 | 10.2±0.9* | --- | --- | --- |
| 8 | --- | 20.0±3.1 | --- | --- |
| 3 | 9.2 | 23.1 | 0.82 | 0.88 |

*Standard error of the mean.

The RQ of the liver, as reported in Table I, compares with that of 0.79 obtained by Gemmill and Holmes (9).

TABLE II.

Oxygen consumption (Q) and RQ of diabetic, male rat liver and kidney.

| Days after alloxan injection. | Liver. Q | Liver. RQ | Kidney. Q | Kidney. RQ |
|---|---|---|---|---|
| 1 | 12.8 | --- | 21.8 | 0.87 |
| 2 | 10.3 | 0.65 | 23.3 | 0.81 |
| 3 | 10.1 | 0.72 | 20.8 | 0.89 |
| 6 | 10.0 | 0.68 | 21.7 | 0.82 |
| 7 | 10.5 | 0.81 | 25.5 | 0.86 |
| 8 | 13.3 | 0.79 | 20.2 | 0.83 |
| 9 | 12.6 | 0.81 | 24.0 | 0.80 |
| 10 | 11.8 | 0.77 | 20.2 | 0.80 |
| 14 | 12.1 | 0.79 | 21.0 | 0.81 |
| 16 | 11.1 | 0.73 | 21.4 | 0.78 |
| 18 | 9.2 | 0.74 | 20.3 | 0.69 |
| 24 | --- | ---- | 23.4 | 0.72 |
| *35 | 12.7 | 0.65 | ---- | ---- |
| 38 | 11.3 | 0.80 | 19.9 | 0.87 |
| 41 | 10.5 | 0.60 | 22.0 | 0.79 |
| 42 | 8.7 | 0.52 | 22.9 | 0.84 |
| Average. | 11.1 | 0.72 | 21.9 | 0.81 |
| Avge. after * |  | 0.64 |  | 0.83 |

The alteration in oxygen consumption is not statistically significant. The kidney RQ in the diabetic animal is lower than normal. The liver shows the greatest change dropping from a normal RQ of 0.82 to 0.64. Other workers have obtained similar results in liver and kidney from depancreatized cats. Fazekas et al (8) reported a kidney RQ of 0.67. The same workers, in another paper (11), reported a liver RQ of 0.59. In the case of liver they stated that, because of the reduced oxygen consumption of the fatty tissue, it was necessary to use more tissue. They got oxygen consumption values which were half their normal values. Their lower result was undoubtedly due to the increased fat content of the liver.

Before evaluating the results it was necessary to find whether the RQ could be varied by dietary changes.

### 2. Effect of Starvation.

TABLE III.

Oxygen consumption (Q) and RQ of liver and kidney of normal, starved rats.

| No. rats. | State. | Liver. Q | Liver. RQ | Kidney. Q | Kidney. RQ |
|---|---|---|---|---|---|
| 3 | Normal | 9.2 | 0.82<br>(0.79-0.83) | 23.1 | 0.88<br>(0.87-0.88) |
| 1 | 24 hrs. fast. | 9.4 | 0.68 | 26.5 | 0.83 |
| 5 | 48 hrs. fast | 8.3 | 0.55<br>(0.52-0.59) | 22.9 | 0.79<br>(0.76-0.82) |
| 1 | 72 hrs. fast | 8.1 | 0.62 | 19.2 | 0.77 |

It will be noted from Table III that the RQ of the liver of starved animals does decrease markedly from a normal of 0.82 to 0.68 in 24 hours to 0.55 after 48 hours. During the same time the oxygen consumption drops only slightly. This agrees with Marsh (15) who obtained an RQ of 0.56 - 0.60.

### 3. Effect of Diet.

(a) 100% sucrose diet: In an attempt to determine what dietary factor would restore the RQ after starvation, sucrose was fed to normal animals which were first starved for 48 hours. Animals were chosen whose weights were between 275 and 305 grams.

TABLE IV.

Oxygen consumption (Q) and RQ of liver and kidney of normal rats on 100% sucrose diet.

| Days on diet | Liver Q | Liver RQ | Kidney Q | Kidney RQ | OW | WAF | WWK |
|---|---|---|---|---|---|---|---|
| 2 | 6.1 | 0.91 | 23.6 | 0.82 | 288 | 250 | 253 |
| 3 | 4.1 | 0.89 | 18.9 | 0.79 | 285 | 244 | 245 |
| 12 | 4.6 | 0.65 | -- - | - -- | 282 | 240 | 212 |
| 18 | 4.3 | 0.65 | 20.9 | - -- | 303 | 265 | 214 |

OW = original weight of rats.
WAF = weight of rats after fast.
WWK = weight of rats when killed.

As seen in Table IV, the RQ was restored by the sucrose diet and even increased over normal on the second and third day. However after the third day the RQ was depressed below normal. An explanation for this probably lies in the weight changes of the animals. For two to three days the animals maintained their weight on this diet but after this time they lost weight which brought the RQ down towards starvation levels again.

(b) Fat-free diet: Since no conclusions can be drawn from an experiment in which the animals lose weight the following diet was devised on which the animals could maintain their weight;

| | |
|---|---|
| Sucrose . . . . . . . . | 70% |
| Casein . . . . . . . . | 25% |
| McCollum's salt mixture . . | 4% |

Vitamins were added as follows (per Kg. of diet);

| | |
|---|---|
| Thiamin . . . . . . . . | 3.6 mg. |
| Riboflavin . . . . . . . | 6.0 mg. |
| Nicotinic acid . . . . . | 36.0 mg. |
| Choline chloride . . . . | 1.0 g. |

All rats were starved 48 hours before they were placed on this diet. Serum Phosphatases were done on some animals as indicated in Table V and from past work they should all be low. The experiment was done twice. Results, placed in order of grams of diet consumed, are combined in Table V.

Glycolysis studies were done since it was thought that they might provide a better explanation for phosphatase results. According to a table given in Hawk, Oser and Summerson (10) the normal anaerobic glycolysis for liver is about 3.0 μl. .

TABLE V.

Oxygen consumption (Q), RQ and anaerobic glycolysis (G) of liver of normal rats on a fat-free diet. One rat used for each row of values.

| Days on diet. | C. | P. | Liver Q | Liver RQ | Liver G | OW | WAF | WWK | AL |
|---|---|---|---|---|---|---|---|---|---|
| 0 | --- | --- | 5.8 | 0.58 | 0.76 | 303 | 276 | --- | N |
| 2 | 33 | 46.0 | 5.3 | 1.1 | 7.6 | 290 | 263 | 295 | L |
| 3 | 60 | --- | 6.7 | 0.72 | 5.1 | 285 | 252 | 280 | L |
| 5 | 70 | 44.8 | 8.4 | 0.68 | 3.4 | 294 | 263 | 300 | L |
| 5 | 83 | --- | 5.9 | 0.54 | 5.8 | 287 | 250 | 288 | L |
| 8 | 87 | --- | 7.7 | 0.87 | 5.6 | 283 | 261 | 289 | N |
| 7 | 127 | --- | 6.9 | 0.89 | 3.1 | 283 | 242 | 280 | N |
| 10 | 145 | --- | 7.4 | 0.90 | 7.7 | 291 | 254 | 300 | N |
| 15 | 182 | --- | 8.1 | 0.79 | --- | 268 | 245 | 276 | N |

C = consumption of food in grams.

P = serum alkaline phosphatase(units/100 ml.)
OW = original weight of rats.
WAF = weight of rats after fast.
WWK = weight of rats when killed.
AL = appearence of liver.
N = normal
L = large

On this fat-free diet the animals regained, in two days, the weight lost during the 48 hour starvation period. The RQ varies as on the 100% sucrose diet, i.e. it increased for two days and then dropped. However after seven to eight days on the diet or after they had eaten about 85 g. the RQ rose sharply to approximately 0.90. From this time on the RQ was slightly above normal. With this change there occurred a physical change in the size of the liver. After two days on the diet the liver, which was normal in size after starvation, was greatly increased in size. The liver returned to its normal size when the RQ increased. These changes must be related to some adaptation to the diet.

According to Cori (7), in a normal fasted rat about 50% of the absorbed glucose is deposited as glycogen in the liver and muscle while the rest is oxidized. However once the glycogen stores are replenished a large part of the absorbed glucose may be deposited as fat. This is probably what occurred and the initial decrease in RQ, and increase in the size of the liver, was due to glycogen being deposited. After the glycogen reserves had been replenished part of the glucose was converted into fat, which was indicated by the increase in RQ.

Burr and Beber (3) in metabolism studies on fat deficiency in rats reported that fat deficient rats have a

higher than normal RQ. They found that the RQ of fat deficient rats was over 1.0 for as long as 12 out of 24 hours, indicating synthesis of large amounts of fat.

After 48 hour starvation, the glycogen stores had been depleted (7) and as would be expected, glycolysis was low. On this diet glycolysis was higher than normal which could mean an increase in enzymes concerned with phosphorylating compounds (eg. phosphatase). However these results are somewhat erratic and do fall to normal in some animals.

## DISCUSSION.

The RQ of liver and kidney is lower in alloxan diabetic rats than in normal animals. However, it is not low enough to indicate gluconeogenesis from fat, although it could indicate gluconeogenesis from protein since the theoretical conversion of protein to carbohydrate calculated by various workers varies between 0.61 and 0.70 (17).

Stetten and Klein (18) found that in alloxan diabetic rats about 75% of urinary glucose was of dietary origin and only 25% represented gluconeogenesis. Cori (7) believes that certain amino acids undergo gluconeogenesis since there is an increased nitrogen excretion in the urine of depancreatized dogs.

Gemmill and Holmes (9) found that the RQ of rats fed butter for two days averaged 0.58. This decrease as well as that noted in starvation might be accounted for by the excessive ketogenesis which does take place in both cases.

The failure of lipogenesis in the alloxan diabetic rat has been reported by Stetten and Klein (19) who found a large increase in the rate of hepatic lipogenesis when insulin was injected. This decrease is probably another indication of the general impairment of glucose utilization

in the diabetic animal.

Bach and Holmes (1) demonstrated on liver slices, in vitro, that insulin inhibited deamination. This too, is probably an indirect effect since Cori (7) claims that the fate of reactive two-carbon fragments formed from fatty acids in the liver depends on the availability of $C_4$-dicarboxylic acids. In their absence fragments condense with each other to forme acetoacetate, in their presence the $C_2$ intermediate is oxidized. This oxidation is coupled with the uptake of inorganic phosphate and the regeneration of ATP. The anti-ketogenic mechanism of the liver depends on three reactions which lead to formation of dicarboxylic acids. These three reactions are;

1. pyruvate + $CO_2 \xrightarrow{\qquad}$ oxaloacetate
2. transamination between pyruvate and either glutamate or aspartate.
3. Direct deamination of these two amino acids.

If the first two reactions are at a minimum it is possible that the last reaction is speeded up and this could account for the increased deamination of protein in diabetes.

It is possible that excessive ketogenesis combined with increased protein deamination could account for the decreased RQ in diabetic animals. The normal fasting animal produces ketone bodies but may not be able to oxidize them as well as the diabetic animal which has more available protein to be deaminated to produce $C_4$-acids necessary for the complete oxidation of fats.

## SUMMARY.

1. The endogenous respiration is the same in normal and alloxan diabetic liver and kidney tissue*.

2. The RQ of the liver and kidney tissue in alloxan diabetic rats is significantly lower than in normal animals*.

3. This lowered RQ is not low enough to indicate gluconeogenesis from fat but could indicate conversion from protein.

4. The RQ of a 48 hour starved rat's liver is significantly lower than normal*.

5. An important observation is that the RQ of the liver of animals on a fat-free diet is higher than normal for two days, lower than normal for five days, and then somewhat higher than normal. This could indicate lipogenesis.

6. With the exception of the starved animal there seemed to be an inverse relationship between the RQ of the liver and the serum alkaline phosphatase activity.

* These results have been reported earlier by other authors.

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# SECTION II.

# OXYGEN CONSUMPTION OF MAMMARY TISSUE IN CANCER SUSCEPTIBLE MICE.

## INTRODUCTION.

It has been known for a number of years that there is a difference in the enzymatic pattern in respiration between tumors and normal tissues. Warburg (17) reported the difference in the glycolysis of normal and cancerous tissue. Since then many workers have studied the enzyme systems of tumors and compared them with their normal tissue of origin. Much of this work has been review by Greenstein (6) who points out that one outstanding result of the tumor process is that the reserves of respiratory enzymes, particularly iron containing ones, are depressed. He also notes that in many cases of neoplastic disease accompanied by severe secondary anemia considerable amounts of iron as well as copper may accumulate in both liver and spleen. Even when anemia does not exist, such storage frequently takes place.

Rawlinson (10) has reported differences in the iron content of mammary glands of high and low strain cancer mice. He reported iron deposition in the glands of high strain cancer mice (dba) at 20 weeks. Within the tumors from these mice at a later age there was no evidence of iron.

It was hoped that these changes in iron content might be reflected in the activity of the iron-containing enzymes. Of the iron-containing enzymes by far the most

important is cytochrome oxidase and with it the various cytochromes which it oxidizes. There is some dispute in the literature as to what percentage of respiration is carried on through this system. To determine this cyanide has been used. This enzyme inhibitor forms a complex with cytochrome oxidase (Fe-CN) thereby inactivating it and consequently the entire cytochrome system.

In a review of the cyanide inhibition of cell respiration, Commoner (2) concludes that 90% of the respiratory rate of most active aerobic tissues and organisms is due to the Warburg - Keilin system. This system involves;

$$\begin{tikzcd}[row sep=small]
\text{Substrate metabolite} \arrow[d] & \\
\text{dehydrogenase} \arrow[d] & \\
\text{cytochrome complex (a, b, c,)} \arrow[d] & \\
\text{cytochrome oxidase } (Fe^{++} \text{ to } Fe^{+++}) \arrow[dr] & \\
\text{atmospheric oxygen} \arrow[u] & \text{water}
\end{tikzcd}$$

The cytochrome complex refers to cytochromes a, b, and c but since little is known about the function of either a or b, it referes chiefly to cytochrome c. Commoner identifies the cyanide stable respiration as being due to flavoproteins.

Rosenthal and Drabkin (12) in a survey of the cytochrome c content of normal and neoplastic epithelium state that along with certain other tissues mammary gland

has a low cytochrome content and that benign and malignant neoplasms derived from this group of tissues do not contain significantly smaller amounts of this respiratory pigment than their parent tissue. However their results were expressed as $\gamma$ of cytochrome c/ gram dry weight of tissue. On this basis there is a considerable error as the following work shows, since mammary gland contains a large quantity of fat which is almost completely absent in the tumor.

## METHODS.

The Test Animals: Male albino rats (Wistar Strain) were used to establish methods. The methods in general follow those presented in Section I, all changes are noted where necessary. These methods were used on $C_3H$ mice in which the incidense of adenocarcinoma (mammary gland tumors) is 63% in virgin and 95% in breeder females. Studies were made on $C_{57}$ (cancer resistant strain) but numbers were insufficient to report.

### The Effect of Calcium Ions in The Medium.

The usual Ringer's solution contains sodium, potassium and calcium ions and is isotonic with the plasma of the animal being used. (See SectionI for constituents of Ringer's solution.) It was found that when phosphate buffer was added the resulting solution was not stable for any length of time. In an effort to maintain the stability of the medium, calcium ions were left out. When calcium was omitted no precipitate of calcium phosphate formed. The following experiment was done to see whether the deletion of calcium ions affected the respiration*. The experiment was done in triplicate on rat liver. The results were as follows;

Oxygen consumption with calcium ions in the medium = 7.0 µl.
Oxygen consumption without calcium ions in the medium = 7.1 µl.

* Endogenous oxygen consumption.

Since calcium was apparently not required it was omitted from the medium in all the work reported in Sections II and III.

The medium used for the work reported in these sections was Ringer-phosphate-glucose, unless otherwise specified, which except for the absence of calcium chloride was the same as that given in Section I. Thus the Ringer solution contained;

| | |
|---|---|
| 0.9% NaCL . . . . | 98 parts. |
| 1.15% KCl . . . . | 2 parts. |

The Use of Succinate.

Succinate was chosen as a substrate for the cytochrome system since it has been established that succinic dehydrogenase is oxidized by this system.

eg. succinate $\xrightarrow[\text{succinic dehydrogenase}]{-2\text{ H}}$ fumarate

$$\begin{tikzcd} \text{succinic dehydrogenase} \arrow[d] \\ \text{cytochrome complex} \arrow[d] \\ \text{cytochrome oxidase etc.} \end{tikzcd}$$

In most tissues, for example liver, there is sufficient succinic dehydrogenase so that the limiting factor is the cytochrome system.

Since there is no agreement in the literature as to the optimum concentration of this substrate it was necessary to select one concentration, of the several reported.

Among others, Canzanelli et al (1) used 0.01 M and Craig et al (3) used 0.02 M. The following experiment was run to determine which was the more suitable concentration.

Three different concentrations of succinate were prepared as follows; 0.2 M succinic acid was brought to a pH of 7.4 with normal NaOH. The solution was then diluted to 0.1 M. 0.2 ml. was added to 1.8 ml. of Ringer-phosphate during the experiment so that the final concentration was 0.01 M. Similarly 0.15 M and 0.20 M solutions were prepared. The oxygen consumption was measured as previously detailed in Section I except for the following changes;

1. The manometers were read until a steady endogenous respiration was measured, usually 30 minutes.
2. The succinate solution was then added by tipping in from the side-arm.
3. The first five minute reading was disregarded and then the manometers were read at five minute intervals for 15 - 20 minutes. The oxygen consumption was "linear" for this length of time.

The results reported in Table I (next page) are the averages of duplicate determinations on rat liver slices. From the results the most suitable concentration of succinate is seen to be 0.02 M for approximately 150 mg. dry weight of liver.

TABLE I.

The effect of various concentrations of succinate on the respiration of rat liver slices.

| M | DW | Q | S | % |
|---|---|---|---|---|
| 0.01 | 0.0140 | 7.6 | 14.3 | 90 |
| 0.015 | 0. 0160 | 7.8 | 19.2 | 146 |
| 0.02 | 0.0160 | 7.3 | 18.1 | 148 |

M = molarity of succinate.

DW = dry weight of liver slices(g.).

Q = oxygen consumption in μl./hr./mg. dry tissue.

S = oxygen consumption after the addition of succinate.

% = percent increase of $\frac{S-Q}{Q} \times 100$

It was decided later that there would be a smaller percentage of substrate left in the side-arm if 0.3 ml. of 0.14 M succinate were added to 1.8 ml. Ringer-phosphate. 2.5 N NaOH was used to neutralize this substrate.

Effect of pH.

Greenstein (6) mentions that one of the characteristics of hepatoma is that when excess glucose is added the pH falls from 7.0 to 6.4 due largely to lactic acid formation. This is in contrast to normal liver the pH of which in the presence of excess glucose does not change from the normal of 7.4.

It was thought that it would be interesting to test the effect of varying the pH on the oxygen consumption and on the response to succinate. The results shown in Table II were done on rat liver slices, in duplicate on rats # 1 and # 2 only. Ringer-phosphate was used as the medium. Phosphate buffer was made up to the required pH.

TABLE II.

The effect of varying the pH on the oxygen consumption of rat liver slices.

| Rat No. | Oxygen Consumption. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| pH | 6.0 | 6.2 | 6.4 | 6.6 | 7.0 | 7.4 | 7.8 | 8.2 |
| 1 | --- | --- | --- | 4.6 | 5.5 | 6.3 | --- | --- |
| 2 | --- | --- | --- | 6.1 | 7.5 | 8.2 | --- | --- |
| 3 | 5.0 | --- | 4.7 | --- | --- | 7.1 | --- | --- |
| 4. | --- | 5.2 | --- | 6.2 | 6.2 | 7.0 | 7.8 | 8.0 |

TABLE III.

The effect of varying the pH on the response to succinate(S)(μl.) by rat liver.

| pH | Q | S | Increment. |
|---|---|---|---|
| 6.0 | 5.0 | 11.3 | 6.3 |
| 6.4 | 4.7 | 12.7 | 8.0 |
| 7.4 | 7.1 | 17.2 | 10.1 |

Q = oxygen consumption in μl./hr./mg. dry tissue.

In all cases a pH below 7.4 resulted in lower oxygen consumption. In two cases a pH above 7.4 resulted in higher respiration. At pH 7.4 the ability of the tissue to increase its respiration above the endogenous value when succinate is added is approximately twice that at pH 6.0. This is seen in Table III. At pH values below 7.4 either succinic dehydrogenase or the cytochrome system or both are adversely affected.

## The Use of para-Phenylenediamine.

Keilin and Hartree (7) have used para-phenylenediamine (ppd) as a substrate for the cytochrome system since they described its effect in 1938. Slater (15) concluded that ppd rapidly reduces cytochrome c in heart muscle preparation and that the preparation does not possess a pathway for its oxidation additional to the cytochrome system.

The control and experimental flasks were prepared as follows; 0.3 ml. of 1.4% freshly prepared ppd was brought to a pH of 7.4 with 0.1 N HCl and added to the side -arm of the flasks into which 1.8 ml. RPG had been placed. (Final concentration = 0.2%) The manometers were read as under Succinate (page 29).

## Preparation of Mammary Gland for Respiratory Studies.

When the following work was started it was pointed out that mammary gland contains a large percentage of fat.

It was decided that the results would be more accurate if they could be based on dry, fat-free weight.

After trying other methods the following procedure was decided upon. The mice were killed by decapitation and the mammary tissue was removed at once, cut into small pieces with scissors and placed in the reaction vessels. After the experiment the pieces were transfered to Gooch crucibles with watch glasses underneath them and placed in a 110°C oven overnight. They were weighed, then ether was added and they were boiled to remove the bulk of the fat. The remaining fat was removed in a Soxhlet apparatus and then the crucibles were again weighed. The two inguinal glands were used since they contain less muscle than the fore-glands. The complete gland was used in one flask. The liver of each animal was run as a control.

## EXPERIMENTAL.

### The Effect of Sex and Age.

It was necessary to determine whether or not there was any change in the activity of the cytochrome system in the mammary gland before the appearence of the tumors. It was decided that the mice would be studied at ten week intervals starting at approximately 15 weeks when the mice should be mature. This work was not completed due to sickness in the mouse colony. The results that were obtained are presented in Tables IV and V.

Histological iron estimations were done by Dr. Rawlinson on the fore-glands. The glands were graded from A to D with those graded A having little iron and those having a great deal of iron graded D.

Histological iron appears in the mammary glands of $C_3H$ female mice at about 15 weeks. Male glands never contain histological iron. However, total iron estimations reveal the presence of iron in male glands but to a lesser extent than in females (11).

The response to ppd was always measured on the left gland and the response to succinate on the right gland. The value obtained for the endogenous oxygen consumption of one gland does not necessarily agree with that obtained on

the other gland. For this reason the endogenous oxygen consumption should always be measured on each gland.

### TABLE IV.

Response to ppd and succinate by normal $C_3H$ female mammary glands at various age intervals.

| No. | Fe | Age | V or B | Q | Av.Q | P | Av.P | Q | Av.Q | S | Av.S |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 409 | B | 15 | V | 9.1 | | 54.6 | | --- | | --- | |
| 410 | C | 15 | V | 16.3 | | 44.8 | | --- | | --- | |
| 414 | $A^+$ | 16 | V | 16.1 | 13.8 | 43.8 | 47.7 | --- | | --- | |
| 421 | $A^+$ | 19 | V | 4.9 | | 17.1 | | --- | | --- | |
| 426 | - | 19 | V | 9.6 | 7.3 | 26.8 | 22.0 | --- | | --- | |
| 345 | B | 27 | - | 5.6 | | 20.6 | | 8.3 | | 22.1 | |
| 363 | B | 27 | - | 11.7 | | 20.5 | | 6.2 | | 12.4 | |
| 366 | D | 27 | - | 8.5 | | 25.6 | | --- | | --- | |
| 411* | B | 27 | - | 7.6 | | 21.2 | | --- | | --- | |
| 412* | $A^+$ | 27 | - | 11.1 | 8.9 | 24.3 | 22.4<br>±2.1** | --- | 7.3 | --- | 17.3 |
| 385 | $C^+$ | 33 | - | 7.5 | | 19.4 | | 8.3 | | 18.1 | |
| 386 | - | 33 | - | 7.7 | 7.6 | 17.9 | 18.7 | 6.7 | 7.5 | 13.4 | 15.8 |
| 543 | - | 69 | V | 18.9 | | 32.8 | | --- | | --- | |
| 545 | - | 69 | V | 11.1 | 15.0 | 27.9 | 30.4 | --- | | --- | |

No. = mouse number.
Fe. = histological iron in fore-gland.
Age = in weeks.
V or B = virgin or breeder. "-" referes to probable breeders.
Q = oxygen consumption in μl./hr./mg. dry fat-free weight
P = oxygen consumption after addition of ppd.
S = oxygen consumption after addition of succinate.
* = sick animals ("plague")
** = standard error of the mean
Av.Q = average oxygen consumption for each group.
Av.P = " " " " " after adding ppd.
Av.S = " " " " " after adding succinate.

Male glands were pooled because of their small size. Two to three left inguinal glands were used for ppd. and the corresponding right glands for succinate.

TABLE V.

Response to ppd (P) and succinate (S) by normal $C_3H$ male mammary gland at various age intervals.

| No. | Age. | Q | P | Q | S |
|---|---|---|---|---|---|
| 2 | 8-10 wks. | 6.5 | 30.1 | --- | --- |
| 2 | 15-16 wks. | 6.3 | 31.6 | --- | --- |
| | Average. | 6.4 | 30.9 | | |
| 17 | 25-30 wks. | 6.0 | 16.9±1.8 | 5.2 | 10.6±3.4 |

No. = number of mice in each age group.

The results of these experiments are summarized in Table VI.

TABLE VI.

Summary of results on normal, $C_3H$ male and female mammary gland.

| Age. | Female | | Male | | Female | | Male | |
|---|---|---|---|---|---|---|---|---|
| | Q | S | Q | S | Q | P | Q | P |
| Up to 16 wks. | --- | --- | --- | --- | 13.8 | 47.7 | 6.4 | 30.9 |
| 19 wks. | --- | --- | --- | --- | 7.3 | 22.0 | --- | --- |
| 25-30 wks. | 7.3 | 17.3 | 5.2 | 10.6 | 8.9 | 22.4 | 6.0 | 16.9 |
| 30-35 wks. | 7.5 | 15.8 | --- | --- | 7.6 | 18.7 | --- | --- |
| 69 wks. | --- | --- | --- | --- | 15.0 | 30.4 | --- | --- |

The following statistically significant differences were found;

1. There was a sex difference in the response of the inguinal mammary glands of $C_3H$ mice to ppd at 16 weeks and at 25 - 30 weeks. There was also a sex difference in the response to succinate at 25 weeks.
2. There was a decrease in the response to ppd by both males and females between 16 and 19 weeks. In the females there seemed to be a further drop between 30 to 35 weeks. At 69 weeks there was an increase in response to ppd to a value exceeding that found at 25 weeks and equal to that found on male mice before sexual maturity.
3. There was a decrease in the endogenous respiration of the female glands after 16 weeks. After the glands involuted the respiration returned to the value obtained before sexual maturity, ie. before 16 weeks.

Since histologically there is a ~~no~~* mammary gland in the male mouse (14) if the value obtained on male mice is subtracted from that of female mice the difference should represent glandular respiration. Considering the results at 25 - 30 weeks only, for succinate this value is 6.7 ul. and for ppd is 5.5 ul. . The same difference between male and female glands was therefore found with both substrates.

---

* rudimentary

Since the response to succinate was lower than that to ppd it must be concluded that succinic dehyrogenase is a limiting factor and therefore succinate should not be used to measure the cytochrome system in mammary gland. This possibility is also mentioned by Craig et al (3).

The non-glandular respiration is probably due to the lymph node which is always part of the material dissected out. This should be proved by dissecting out the lymph node and determining its respiration as well as the glands.

## The Effect of Pregnancy and Lactation.

Since pregnancy and lactation would be expected to change the respiration of mammary gland a study of these two conditions was warranted. Histological iron decreases during pregnancy and disappears during lactation (10). On account of sickness in the mice colony it was possible to study pregnancy only. These results are presented in Table VII.

The wet weight, dry weight and fat-free weight figures are included in Table VII. The amount of fat-free material had doubled by the end of pregnancy from the normal of 50 mg. (at 25 - 30 weeks) to over 100 mg. . The wet weight had also increased. These figures are further

considered in Table IX.

TABLE VII.

The effect of pregnancy on the response to ppd and succinate of mammary gland.

| No. | Fe | F | Q | P | FFW | DW | WW | Q | S |
|---|---|---|---|---|---|---|---|---|---|
| 346 | $B^+$ | 5 | 5.1 | 24.4 | 0.007 | 0.05 | 0.17 | 12.9 | 19.9 |
| 360 | B | 12 | 12.2 | 24.4 | 0.006 | 0.04 | 0.13 | 19.8 | 25.8 |
| 359 | $B^+$ | 19 | 15.4 | 25.7 | 0.011 | 0.07 | 0.28 | 15.3 | 18.8 |
| 362 | D | 19 | 15.4 | 22.8 | 0.013 | 0.07 | 0.29 | 13.1 | 13.6 |
| 353 | B | 21 | 4.4 | 20.0 | 0.010 | 0.05 | 0.19 | 18.0 | 18.0 |
| Avge. | | | 10.5 | 23.5 | | | | 15.8 | 19.2 |

NO. = number given animal. All animals 25-30 weeks.
F = length of fetus in mm. .
FFW = fat-free weight of mammary gland.
DW = dry weight of mammary gland.
WW = wet weight of mammary gland.

If the two "Q" values in Table VII are averaged (the right and left glands) the endogenous respiration was 13.2 ul. which represents an increase over the normal of 8.4 ul. (the average of the right and the left glands at 25 -30 weeks). The response to ppd and to succinate was the same as in normal mice of the same age.

## The Effect of Tumors.

A study of the respiration of tumor tissue was done on a few mice. All tumors except that from mouse # 542 were full of blood. Mouse # 542a was included with these results because there seemed to be small cysts in the fore-glands. The respiration of the inguinal glands from the same animal was also studied. The results of these experiments are presented in Table VIII. As mentioned earlier the response to succinate was studied with the right inguinal glands and the response to ppd with left glands.

In the case of mouse # 361 both the tumor and the gland response to ppd was about half the normal values for this age group, of 22.4 ul. . The tumor contained no fat and the gland much less fat than normal which is 20 - 30% of the wet weight.

The tumor response to ppd was low while the gland of # 365 did not seem to be affected. The fat percentage was low in the tumor and normal in the gland.

The tumor tissue of # 377 did not respond to ppd. Both the gland and the tumor response to succinate was lowered. The response to ppd by the gland was normal. Fat percentage was low in both the tumor and the gland.

TABLE VIII.

The response to ppd and succinate of tumors and inguinal glands from $C_3H$ female mice.

|  |  |  | GLAND |  | TUMOR |  | GLAND |  | TUMOR |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Age | Fe | Q | S | Q | S | Q | P | Q | P | FG | FT | Size |
| 361 | 25 | * | ---- | ---- | --- | ---- | 8.2 | 13.6 | 9.0 | 12.0 | 3% | 0.0% | F3 |
| 365 | 26 | C | 17.9 | 19.9 | --- | ---- | 6.9 | 25.1 | 9.9 | 12.7 | 30% | 5.0% | IL 1/2 |
| 377 | 30 | D | 8.1 | 12.9 | 8.4 | 9.2 | 6.0 | 20.8 | 7.1 | 6.8 | 7% | 1.0% | FR3 |
| 384 | 31 | $B^-$ | 7.1 | 15.9 | 8.8 | 10.1 | 3.4 | 13.6 | 10.7 | 11.1 | 10% | 0.5% | FL3 |
| 387 | 32 | C | 10.3 | 18.3 | 9.7 | 10.7 | 7.3 | 17.6 | 9.7 | 13.3 | 15% | 2.0% | FL2 |
|  |  |  | 10.4 | 16.8 | 9.0 | 10.0 | 6.4 | 18.1 | 9.3 | 11.2 |  |  |  |
| 542 | 63 | - | ---- | ---- | --- | ---- | 6.9 | 17.2 | 14.0 | 18.2 | --- | ---- | FL |
|  |  |  |  |  |  |  | 9.7 | 27.1 |  |  |  |  |  |
| 542a | 63 |  | ---- | ---- | --- | ---- | 7.6 | 16.4 | ---- | ---- | --- | ---- | --- |

Fe = iron in fore-glands.
FG = percent fat in gland.
FT = percent fat in tumour.
Size = size and location of tumour.    F = fore-gland.    I = Inguinal.    R = Right
L = Left.    2, 3 etc. = Diameter of tumour in cms.

[illegible]

With # 387 and # 384 there was a low response to ppd and succinate by the tumor. The gland tissue response to ppd was lower than normal while its response to succinate was normal. There was a lowered percentage of fat in the tumor and in the gland.

In the case of mouse # 542 the tumor response to ppd was lower than the normal value of 30 ul. but higher than that found with the other tumors. The inguinal gland on the same side of the body showed a lowered response to ppd. The glands of mouse # 542a also showed a decreased response to ppd.

These results can be summarized as follows;

1. The tumor response to ppd and succinate was significantly lower than normal.
2. The percentage of fat in the tumors was much less than normal.
3. A large tumor (2 - 3 cm.) affected the respiration of the gland on the same side of the body as the tumor.
4. If the histological iron in the gland had been decreased the response to ppd by the gland on the same side of the body as the tumor, was the same as the tumor.
5. The endogenous respiration was about the same in the gland and in the tumor.

Hypervolemia (increased blood volume) and anemia were noticed* in the mice that bore tumors. Enlargement of the liver and spleen were also noticed. Hypervolemia has also been reported by Sobel and Furth (16). They noticed that the blood volume began to rise after tumors (granulosa cell growths) reached a moderate size. The greatest portion of the increased blood volume was in the liver. The spleen and adrenals were also affected. In the liver branches of the portal vein and sinusoids were greatly dilated.(5).

### The Effect of Various Factors on the Percentage of Fat in the Mammary Gland.

During these experiments certain changes in the percentage of fat in the gland were noted. These results are presented in Table IX.

There was an increase in the dry, fat-free weight and a decrease in the fat content after 16 weeks in the mammary glands of female mice. The percentage of fat was decreased by sickness and by tumors. It was not affected by pregnancy.

---

* This was noted when the blood was collected for other experiments.

TABLE IX.

The fat content of the mammary gland of female and male mice under various conditions.

| No. and Type. | FEMALE Age | Fat* | FFW | Fat** | MALE No. | Fat* | FFW |
|---|---|---|---|---|---|---|---|
| 3 | 15-16 | 34% | 2% | 95% | 2 | 20% | 2% |
| 3 | 25-30 | 20% | 5% | 80% | 17 | 30% | 3% |
| 6-P | 25-30 | 24% | 4% | 86% | -- | --- | -- |
| 1-S | 25-30 | 8% | 3% | 73% | -- | --- | -- |
| 5-T | 25-35 | 13% | 4% | 66% | -- | --- | -- |

Fat* = percent fat of wet weight.
Fat**= percent fat of dry weight.
FFW = percent fat-free weight of wet weight.
P = pregnant.
S = sick (plague).
T = bearing tumors.

There was less fat in the tumors than in normal mammary glands. The average percentage of fat, for 5 tumors, was 2% of the wet weight and 17% of the dry weight. There was also a higher percentage (average = 8%) of fat-free weight in the ~~gland~~.tumor.

## DISCUSSION.

A lowered response to ppd by the tumors, as compared to normal or pregnant mammary gland, is considered due to a deficiency in either cytochrome oxidase or in cytochrome c. This explanation does not agree with the results obtained by Rosenthal and Drabkin (12) who quantitatively measured the amount of cytochrome c in pregnant mammary gland and spontaneous adenoma. However, as mentioned in the introduction their results were based on dry weight and since our work has shown that the dry weight contains a considerable percentage of fat their results are misleading. Using the percentage fat of the dry weight that we found for pregnant and tumor tissue, Rosenthal and Drabkin's results have been recalculated in Table X on a dry, fat-free weight basis. This has been done for the sake of comparison only since we cannot say that a lowered percentage of fat is characteristic of all types of mammary gland tumors.

On a fat-free, dry weight basis it is seen that the pregnant gland actually contains much more cytochrome c than spontaneous adenoma. These recalculated results for the two tissues compare favorably with the response to ppd that was obtained by us.

TABLE X.

Results of Rosenthal and Drabkin on the cytochromec content of pregnant mammary gland and adenoma; results recalculated from a dry weight to a dry, fat-free weight.

| | 1. | 2. | 3. |
|---|---|---|---|
| Mammary gland of 14-day pregnant mouse. | 32 | 86% | 228 |
| *Spontaneous adenoma. | 51 | 17% | 62 |

1.= Results obtained by Rosenthal and Drabkin expressed as γ of cytochrome c / gm. dry weight.

2.= Percent fat from our previous experiments.

3.= Rosenthal and Drabkin's results recalculated to γ of cytochrome c / gm. dry, fat-free weight.

*= slow growing.

The tumor is also deficient in succinic dehydrogenase. The similarity of our results to results obtained by Craig et al (3) on mice hepatoma is striking. They used o.02 M succinate and their results, along with ours, are reported in Table XI.

see Table XI on next page

TABLE XI.

A comparison of our results on the response to succinate on mammary gland and tumor with those obtained by Craig et al on liver and hepatoma.

| Investigator. | Organ. | Q. | S. | $\frac{S-Q}{Q}$ x 100 |
|---|---|---|---|---|
| Craig et al. | Normal liver. | 10.6 | 25.2 | 137% |
| | * Hepatoma | 10.5 | 12.2 | 17% |
| This lab. | Normal mam.gland | 8.4 | 17.3 | 106% |
| | Adenocarcinoma | 9.0 | 10.0 | 11% |

* Hepatoma produced by feeding butter yellow.

The question as to whether or not any change in the cytochrome system occurs before malignancy has been studied by a number of workers who do not agree on this point. Mayer (9) using rat liver homogenate response to succinate and ppd found that for the first fifty days after the injection of p-dimethylaminoazobenzene, the cytochrome c activity was high and even increased over normal. After seventy days it started to decline and after 163 days the tumor appeared. Her results are not substantiated by Dubois and Potter (4) who quantitatively measured the cytochrome c content of rat liver for six months before tumors (produced by butter yellow) appeared and found no reduction before the appearance of the tumor. They based their results on

fresh tissue weight.

Our results on mammary gland indicate a drop in the cytochrome system much earlier than has usually been measured. This decrease occurs after sexual maturity and at about the same time as iron-deposition begins. Even if this result were not significant the fact that the presence of a tumor can affect the supposedly normal gland beside it cannot be disputed from the results. The fact that in each case where a decrease in glandular respiration was noted, the deposition of iron was not normal shows that there must be some relationship between the presence of iron and normal gland respiration.

It is difficult to decide which has the most significance, the appearance or the disappearance of the iron. The significance of iron-deposits is hard to evaluate since they also occur in mammary glands of non-cancerous mice and in rats. It has also been found in other epithelial organs. Zondek and Karp (19) investigated the iron-content of epithelial organs of different species at various ages. They found that at a definite period of life (about the middle) and within a short time the iron-content increased 200% in the liver, kidney and testes but not in muscle. They suggested that the iron is in a new compound which was not formally associated with the cell.

The effect of spontaneous mammary tumors on other tissues has been noted by various authors. Warren (18) using male mice injected with dibenzanthracene (has hemolytic action) noted that the iron content of four lymph glands increased up to 20 weeks after the first injection. If the injections were stopped the iron content went down. They also studied some female mice with spontaneous mammary cancer and got the same value as in mice which had been injected.

The possibility that sex hormones are depressing the cytochrome system is suggested by the results. The greatest decrease in the activity of this system occurred after sexual maturity while after the ovaries have degenerated there is an increase. Whether or not this is a natural phenomenon would have to be established before too much importance can be attached to the results. McShan and Meyer (8) found that diethylstilbestrol inhibited cytochrome oxidase in rat liver. They obtained complete inhibition of this enzyme at certain concentrations but were unsuccessful in demonstrating any invivo effect. They suggested that in vivo the estrogen was being inactivated.

The liver results were not included because neither age nor the presence of a tumor affected its

respiration. This agrees with Schneider and Potter (13) who attempted to demonstrate a decrease in activity of the succinoxidase system in livers of tumor bearing animals and were unable to do so. Instead of measuring the response to ppd by liver and basing the results on dry weight, more information might be gained if the total weight of the liver were known so that the total activity of the cytochrome system in the liver could be calculated.

## SUMMARY.

1. There is a sex difference in the response to ppd of the inguinal mammary gland at 16 weeks and at 25 - 30 weeks. There is also a sex difference in the response to succinate at 25 weeks.

2. There is a decrease in the response to ppd by the mammary tissue of both males and females between 16 and 19 weeks. In the females there seems to be a further drop between 30 - 35 weeks. At 69 weeks there is an increase to a value exceeding that found at 25 weeks and equal to that found on male mice before sexual maturity.

3. There is a decrease in the endogenous respiration of the female mammary tissue after 16 weeks. After the glands have involuted the respiration of the gland returns to the value obtained before sexual maturity, i.e. before 16 weeks.

4. Pregnancy does not affect the response by mammary tissue to ppd or succinate but does increase the endogenous respiration.

5. The tumor response to ppd and succinate was significantly lower than normal.

6. The percentage of fat in the tumors is much less than normal.

7. A large tumor affects the respiration of the gland on the same side of the body as the tumor.

8. The endogenous respiration is about the same in the gland and in the tumor.

9. If the histological-iron in the gland has been reduced . the response to ppd by the gland is the same as the tumor.

10. No relationship was found between the quantity of histological-iron present and the activity of the cytochrome system.

11. The significance of these results has been discussed in relationship to related work reported by other workers.

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# SECTION III

## STUDY OF CYTOCHROME SYSTEM IN RAT MAMMARY GLAND.

# INTRODUCTION.

Anumber of workers have tried without much . success to measure the respiration of mammary tissue in experimental animals. Most workers have based their results on dry weight which is the normal procedure for other tissues. (2)(8). Kleiber et al (5) measured the metabolism of rat mammary tissue during pregnancy and lactation. They found that the lactating gland was more active than the pregnant gland if the results were based on dry weight, equally active on the basis of wet weight and that the pregnant gland was the more active if the results were based on mg. of nitrogen. They noted that lactating mammary tissue contained less than half as much dry matter as mammary tissue from pregnant rats. This observation was confirmed by Folley and French (4) who also studied rat mammary gland respiration. They based their results on dry weight and found an increase in the endogenous respiration during lactation as compared to pregnancy. They admit that they are not sure whether or not this is a real increase or is merely an "artifact due to the disappearance of metabolically inert dry matter, such as fat or protein".

## METHODS.

Albino rats (Wistar Strain) were used. They were fed a mixture of Purina and Victor Fox Checkers when not breeding and Purina Fox Checkers when breeding and lactating. The method for measuring the oxygen consumption and the response to ppd by rat mammary gland was the same as that indicated under Section II. The liver of each animal was run as a control.

## EXPERIMENTAL.

Since other workers have based their results on dry weight it was necessary to record this value so that the results might be compared. Because a sex difference had been found in the mice, it was thought a similar difference might exist in rats. For this reason male rats were studied and the results are presented in Table VI. The various conditions which affect the mammary gland were studied in an attempt to learn something about the normal factors which affect its respiration. These conditions included pregnancy, lactation, weaning, castration and the injection of estrogens and the results are presented in Tables I, II, III, IV, V and VII.

## TABLE I.

The oxygen consumption (Q) and response to ppd (P) of normal female mammary tissue.

| No. | Dry Wt. Q | Fat-free Q | Fat-free P | $\frac{P-Q}{Q}\times 100$ | FDW |
|---|---|---|---|---|---|
| 1 | 1.4 | 7.4 | 10.9 | | 82% |
| 2 | 1.0 | 4.8 | 12.2 | | 78% |
| 3 | 0.5 | 5.8 | 8.0 | | 92% |
| 4 | 0.4 | 3.4 | 8.8 | | 90% |
| 5 | 0.3 | 3.1 | 9.1 | | 91% |
| 6 | --- | 4.8 | 8.6 | | -- |
| 7 | --- | 3.5 | 12.5 | | -- |
| 8 | --- | 2.3 | 8.0 | | -- |
| 9 | --- | 1.5 | 10.2 | | -- |
| 10 | --- | 2.0 | 7.7 | | -- |
| 11 | --- | 2.6 | 9.0 | | -- |
| Avge. | 0.9 | 3.7±1.7* | 9.6±1.6* | 160% | 87% |

No. = number of each animal.

Dry wt. Q = oxygen consumption in ul./mg. dry weight/hr.

Fat-free Q = oxygen consumption in ul./mg. fat-free weight/hr.

Fat-free P = oxygen consumption in ul./mg. fat-free/hr. weight after the addition of ppd.

FDW = the percent fat of the dry weight.

* = standard error of the mean.

TABLE II.

The oxygen consumption and response to ppd of pregnant mammary tissue.

| No. | Days pregnant | Dry wt. Q | Fat-free Q | Fat-free P | Inc. | FDW |
|---|---|---|---|---|---|---|
| 12 | 7 | 0.5 | 4.2 | 14.6 | | 90% |
| 13 | 10 | 1.6 | 10.0 | 16.8 | | 83% |
| 14 | 14 | 1.6 | 10.3 | 18.3 | | 84% |
| 15 | 14 | 2.2 | 10.3 | 17.9 | | 80% |
| Avge. | | 1.5 | 8.7 | 16.9 | 95% | 84% |
| Avge.(10-14 days) | | 1.8 | 10.2 | 17.7 | 75% | 82% |

$$\text{Inc.} = \frac{P - Q}{Q} \times 100$$

TABLE III

The oxygen consumption and response to ppd of lactating mammary tissue.

| No. | DAL | Dry wt. Q | Fat-free Q | Fat-free P | Inc. | FDW |
|---|---|---|---|---|---|---|
| 16 | 7 | 2.8 | 7.8 | 10.5 | | 66% |
| 17 | 18 | 6.2 | 11.1 | 12.7 | | 33% |
| 18 | 19 | 4.8 | 10.7 | 11.9 | | 57% |
| 19 | 21 | 7.7 | 10.9 | 14.9 | | 29% |
| 20 | 21 | 5.6 | 10.7 | 15.2 | | 48% |
| 21 | 21 | 1.7 | 7.1 | 10.5 | | 77% |
| 22 | 28 | 3.9 | 6.2 | 9.4 | | 37% |
| Avge. | | 4.9 | 9.2±2.0 | 12.2±2.1 | 33% | |
| Avge.for 18-21 dys. | | 5.2 | 10.1 | 13.0 | 28% | 49% |

DAL = days after birth of litter.

## TABLE IV.

The oxygen consumption and response to ppd of mammary tissue of rats one week after weaning.

| No. | Dry wt.<br>Q | Fat-free<br>Q | Fat-free<br>P | Inc. | FDW |
|---|---|---|---|---|---|
| 23 | 1.1 | 4.7 | 13.2 | | 77% |
| 24 | 0.7 | 5.5 | 16.3 | | 88% |
| Avge. | 0.9 | 5.1 | 14.8 | 190% | 83% |

## TABLE V.

The oxygen consumption and response to ppd of mammary tissue of castrate females.

| No. | Dry wt.<br>Q | Fat-free<br>Q | Fat-free<br>P | Inc. | FDW |
|---|---|---|---|---|---|
| 25 | 0.2 | 3.0 | 13.3 | | 94% |
| 26 | 0.1 | 2.6 | 15.1 | | 98% |
| 27 | 0.4 | 6.3 | 7.8 | | 94% |
| 28 | 0.2 | 3.5 | 9.9 | | 95% |
| Avge. | 0.2 | 3.9 | 11.5 | 195% | 95% |

Table VI on next page.

TABLE VI.

The oxygen consumption and response to ppd of normal male mammary tissue.

| No. | Dry wt. Q | Fat-free Q | Fat-free P | Inc. | FDW |
|---|---|---|---|---|---|
| 29 | 0.4 | 5.4 | 14.5 | | 93% |
| 30 | --- | 4.0 | 8.7 | | --- |
| 31 | --- | 6.2 | 6.7 | | --- |
| 32 | 0.6 | 9.6 | 15.0 | | 93% |
| 33 | 0.9 | 5.9 | 9.2 | | 85% |
| Avge. | 0.6 | 6.2±1.1 | 10.8±3.3 | 74% | 90% |

TABLE VII.

The oxygen consumption and response to ppd of mammary tissue of female rats injected with estrogen.

| No. | Type | Dry wt. Q | Fat-free Q | Fat-free P | Inc. | FDW. |
|---|---|---|---|---|---|---|
| 34 | normal | 0.8 | 5.2 | 11.5 | 120% | 84% |
| 35 | castrate | 0.5 | 2.7 | 8.6 | 218% | 80% |

## DISCUSSION.

On a dry weight basis our results, as summarized in Table VIII, indicate that there is an increase in the endogenous respiration during pregnancy and a much larger increase during lactation. This last increase is due entirely to the decrease in fat content from 82% of the dry weight in pregnancy to 49% of the dry weight in the lactating gland (see Tables II and III). In another sense it may be said that on a dry weight basis there is four times the fat-free weight in the lactating gland compared with glands in pregnant animals. This totally accounts for the difference in endogenous respiration in the glands in the two groups.

TABLE VIII.

A comparison of our results on mammary gland with those obtained by other workers.

| Invest-igator. | Normal | | Preg.10-21 dy. | | | | Lact.15-21 dy. | | | | Wean | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DW | FW | WW | DW | FW | mN | WW | DW | FW | mN | DW | FW |
| Our. lab. | 0.9 | 3.7 | | 1.8 | 10.2 | | | 5.2 | 10.1 | | 0.9 | 5.1 |
| Kleiber et al | | | 0.5 | 0.9 | | 88 | 0.6 | 2.7 | | 31 | | |
| Folley French | | | | 1.3 | | | | 10.3 | | | 5.5 | |

WW = oxygen consumption based on wet weight
DW = " " " " " " dry weight
FW = " " " " " " dry, fat-free wt.
mN = " " " " " " mg. of nitrogen.

Kleiber et al (5) found a three-fold increase and Folley and French (4) reported an eight-fold increase on a dry weight basis in mammary tissue of lactating animals compared with the tissue in pregnancy. However on a fat-free basis there is a three-fold increase over normal in pregnancy which is maintained at a constant level during lactation and then drops seven days after weaning to nearly normal. Because of the diluting effect of fat, which we subsequently show by staining, contains very little cytochrome oxidase, it is felt that our studies reported on a dry, fat-free weight basis give a clearer concept of respiration than is indicated by other workers. It is possible that there is a slight error in the results on lactating glands due to the "milk error" mentioned by Folley (3). It has been assumed that most of the milk has been leached out during the experiment and would not therefore significantly affect the results.

The normal gland uses oxygen at a low rate which can be considerably increased by the addition of ppd indicating that the gland contains considerable cytochrome reserves. These reserves are used during pregnancy and lactation when energy is required for growth and secretion. Some of the increase in total response to ppd during pregnancy and lactation could be due to the fact that there is less connective tissue in these glands than in normal glands.

However the ability to increase their respiration over the endogenous respiration is relative and not influenced by weight. The percentage increases over the endogenous respiration show a successive decrease in the response to ppd from 160% in normal to 95% in the pregnant and 33% in lactating glands. This shows that the pregnant and lactating glands are using their cytochrome reserves practically to the limit and this is especially true for the lactating gland. On weaning the gland respiration decreases and once more the gland has a considerable reserve of cytochrome.

The castrate female glands are very similar to the glands of the normal female. The only difference is a higher percentage of fat in the gland.

That the male rat has a well developed mammary gland has been reported by Schultz (9). This gland is indistinguishable on a respiration basis from a normal female gland but the response to ppd is much more variable and probably depends on the develop~~e~~ment of the gland or on the selection of material for the experiment since the gland is only developed in certain areas.

Estrogen injection has no significant effect on either the endogenous respiration or on the response

to ppd in normal or castrate animals. It does decrease the percent fat in the gland of the castrate female.

## ADDENDUM.

### The Development of a Histological Stain for the Cytochrome System.

It was noticed in oxygen consumption studies with rat mammary gland that the ppd seemed only to be oxidized in certain places. This was indicated by distinctly darkened areas in the tissue. It was thought that the locus of oxidation of ppd might thus be determined histologically.

Other stains for the cytochrome system have been reported. Montagna and Noback (6)(7) reported the use of nadi-reagent (ppd and alphanaphthol) to stain cytochrome oxidase in mast cells. They stated that, other than myeloid elements, few cells in the animal body give a positive reaction to the test. Becker (1) mentioned the nadi-reaction and stated that it did not allow any conclusion as to the localization of cytochrome oxidase.

### Method.

Fresh normal rat mammary tissue was used. The tissue was cut in pieces, placed in Ringer-phosphate-glucose and then ppd (in the same ratio as for manometric determination)

was added. It was then shaken at room temperature for about one hour and left overnight in the reaction mixture. The tissue was washed in distilled water and then 0.1 N HCl was added for about 15 minutes* to leach out the unoxidized ppd. The tissue was then blotted dry and cleared in oil of origanum over night. It was further cleared in xylol and mounted in piccolyte.

## Results.

Slides have been prepared in which the oxidized ppd outlines the gland tree indicating that the cytochrome system is chiefly located in the gland and not in the fat. That the staining is due to the cytochrome system has been proved by the addition of cyanide which completely stops the reaction.

The mammary gland of a fifteen week old female rat, stained by ppd, is shown in Figure I. A section of the same gland, which due to the presence of cyanide did not stain with ppd, was subsequently stained with hemotoxylin to prove the presence of a gland and is shown in Figure II.

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* Leaving overnight does not alter the stained material.

FIGURE I

Inguinal mammary gland of a fifteen week old female rat, stained with ppd, magnification 25 times.

FIGURE II

Section of the same gland which due to the presence of cyanide did not stain with ppd. The section was stained with hemotoxylin to show the presence of a gland, magnification 25 times.

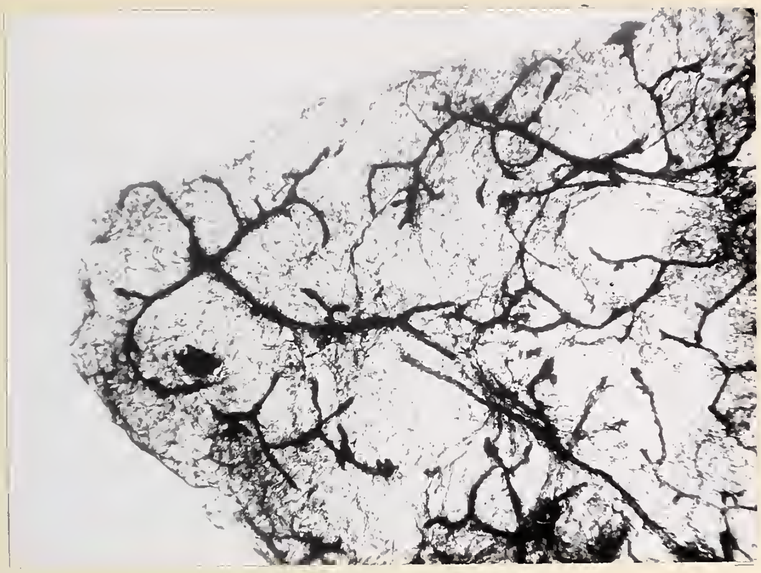

Figure I

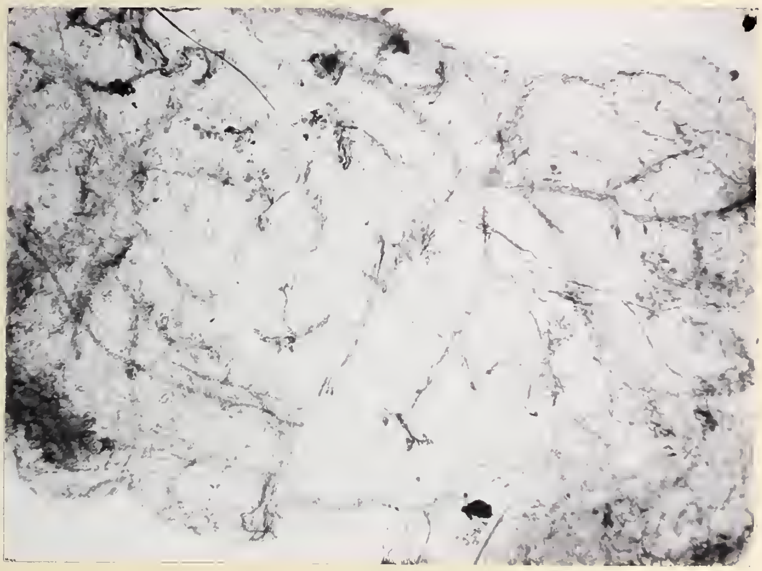

Figure II

# SUMMARY.

1. The normal rat mammary gland contains considerable reserves of the cytochrome system which are used during pregnancy and to a larger extent during lactation.
2. Respiratory sfudies of rat mammary gland when based on dry weight of tissue include a large error due to fat. This factor can only be corrected for by extraction of the fat since the content alters considerably during pregnancy and lactation.
3. The respiration of rat mammary tissue on a dry, fat-free weight basis is increased over normal during pregnancy and lactation and returns towards normal on weaning.
4. There is no significant difference between the response to ppd of normal male and female mammary tissue.
5. Castration increases the fat content of the mammary gland but does not alter its respiration.
6. The injection of estrogens decreases the fat content of the mammary gland of the castrate female but does not alter the respiration of this tissue.
7. The injection of estrogens into normal females does not change the respiration of the mammary gland.
8. A histological stain for the presence of the cytochrome system is presented.

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